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VIP contribution to the decidualization program: regulatory T cells recruitment

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Abstract

During early pregnancy, the human uterus undergoes profound tissue remodeling characterized by leukocyte invasion and production of proinflammatory cytokines, followed by tissue repair and tolerance maintenance induction. Vasoactive intestinal peptide (VIP) is produced by trophoblast cells and modulates the maternal immune response towards a tolerogenic profile. Here, we evaluated the VIP/VPAC system contribution to endometrial renewal, inducing decidualization and the recruitment of induced regulatory T cells (iTregs) that accompany the implantation period. For that purpose, we used an *in vitro* model of decidualization with a human endometrial stromal cell line (HESC) stimulated with progesterone and LPS (Lipopolysaccharide) simulating the inflammatory response during implantation and human iTregs (CD4+CD25+FOXP3+) cells differentiated from naïve T cells obtained from fertile women peripheral blood monuclear cells.

We observed that VIP and its receptor VPAC1 are constitutively expressed in HESC cells and progesterone increased VIP expression. Moreover, VIP induced RANTES expression by HESC, one of the main chemokines involved in T cell-recruitment and this effect is enhanced by the presence of progesterone and LPS. Finally, migration assays of iTregs toward conditioned media from HESC cells revealed that endogenous VIP production induced by P4 and LPS and RANTES production were involved since the anti-RANTES neutralizing Ab or VIP antagonist prevented their migration. We conclude that VIP may have an active role in the decidualization process thus contributing to iTregs recruitment toward endometrial stromal cells by increasing RANTES expression in a progesterone-dependent manner.

INTRODUCTION

Endometrial receptivity, embryo implantation and the maintenance of pregnancy is a sequence of intricate events that requires a coordinated interaction between the endometrial epithelial and stromal cells, the maternal immune system, and the blastocyst (Dey *et al.* 2004, Stoikos *et al.* 2008, Gellerson *et al.* 2007).

In this context, the early pregnant uterus undergoes profound remodeling and leukocyte invasion associated with the production of proinflammatory factors (Pérez Leirós & Ramhorst 2013, Dimitriadis *et al.* 2005) . Under the influence of progesterone, endometrial stromal cells differentiate into epithelioid decidual cells and secrete diverse mediators, which contribute to the generation of a local immune privileged site supporting the nidation of a semiallogenic fetus (Gellerson *et al.* 2003, Mesiano *et al.* 2011).

The decidualization of the stromal cells also occurs in the absence of pregnancy and the declining progesterone levels trigger several effects, such as the expression of proinflammatory cytokines, chemokines and matrix metalloproteinases, and activate a sequence of events leading to menstruation (Salomonsen *et al.* 1999, Catalano *et al.* 2007). Moreover, decidualization in humans is apparent 10 days after ovulation, indicating that progesterone is not the primary trigger of this differentiation process. In fact, initiation of the decidual process is dependent on elevated levels of cAMP (Teklenburg *et al.* 2010a), suggesting that local factors could be involved in the activation of adenylate cyclase in stromal cells.

Since menstruation and early pregnancy are inflammatory conditions that cause a degree of physiological tissue injury, the exposure of the uterus to a threatening stimulus at a dose below the threshold for tissue injury will provide tolerance against a more severe subsequent insult (Teklenburg *et al.* 2010b, King *et al.* 2010).

During the process of tissue renewal associated with the menstrual cycle, uterine cells undergo apoptosis necessary for the removal of cellular debris. Implantation and early placentation represent still another period of high tissue turnover and renewal. During the early stage of implantation, trophoblast cells break the epithelial lining of the uterus in order to adhere, then invade endometrial tissue and

replace endothelial cells, generating apoptotic bodies of trophoblast that will contribute to the induction of a tolerogenic microenvoirenment (Abrahams *et al.* 2004a). Therefore implantation involves a tight homeostatic control provided by immune cells selectively recruited and/or expanded depending on the subpopulation during early stages of gestation and the contribution of redundant molecules able to trigger multiple tolerogenic programs ((Pérez Leirós & Ramhorst 2013, Gomez-Lopez *et al.* 2010). In this context, the modulation of chemokines and their receptors selectively controls the recruitment of different leukocyte populations (Bromley *et al.* 2008, Fraccaroli *et al.* 2009a). During the implantation period, in particular, the β-chemokine CCL5 (RANTES) is locally produced by the human endometrium and, interestingly, it has the potential to act in an autocrine manner by the differential expression of its receptors CCR1, CCR3, and CCR5 (Ramhorst *et al.* 2006, Ramhorst *et al.* 2008). In addition, RANTES is produced by human endometrial T-lymphocytes, CD4+ and CD8+, and its production is increased in the presence of physiological progesterone concentrations (Ramhorst *et al.* 2006).

One of the main effects of RANTES is the induction and the recruitment of regulatory T cells (Tregs) (Fraccaroli *et al.* 2009b). The specialized Tregs population is essential for preventing a maternal immune response against paternal antigens. Basically, natural Tregs (nTregs) (derived from the thymus) that constitutively express CD25 can be distinguished from inducible Tregs, CD4+CD25+ FOXP3+ cells that are induced from CD4+CD25- precursors in the peripheral lymphoid organs (iTregs) (Guerin *et al.* 2009). Prior to implantation the seminal fluid can drive iTregs expansion (Robertson *et al.* 2009). and then the continuous release of placental antigens into the maternal circulation would maintain a Treg population targeted specifically against paternal antigens (Aluvihare *et al.* 2004). Previously, we described the development of an *in vitro* differentiation model of iTregs from naïve CD45RA+CCR7+ obtained from peripheral blood mononuclear cells isolated from fertile women. We observed that trophoblast cells not only contributed to their differentiation in a TGF-β dependent pathway, but also secreted chemokines, such as RANTES, MCP-1, and IL-8, which were capable of selectively recruiting them (Ramhorst *et al.* 2012).

VIP is a pleiotropic peptide with embryotrophic, smooth muscle relaxing, prosecretory and immunomodulatory effects (Ekstrom *et al.* 1983, Spong *et al.* 1999, Covineau *et al.* 2012, Leceta *et al.* 2007, Gonzalez-Rey *et al.* 2007). VIP was shown to down-regulate inflammatory factors and inhibit

antigen specific Th1-driven immune responses switching to a tolerogenic profile with the generation or expansion of Treg cells (Leceta *et al.* 2007, Gonzalez-Rey *et al.* 2007). In addition, among several mediators released locally, we have proposed a role of VIP at the early maternal-placental interface with immunosuppressant and trophic effects (Perez Leiros & Ramhorst 2013, Fraccaroli *et al. et al.* 2009c). Certainly, using an *in vitro* model of trophoblast and maternal leukocyte interaction, VIP showed a Th1-limiting and Treg-promoting response that would favor early pregnancy outcome. VIP also decreased the production of inflammatory mediators after culturing fertile women-PBMCs with trophoblast cells; while it increased TFGβ and IL-10 production (Fraccaroli *et al. et al.* 2009).

Taking into account that endometrial stromal cells are exposed to an inflammatory response that preconditions the uterus at peri implantation period and that VIP mediates pro-tolerogenic responses, we evaluated the contribution of VIP/VPAC system to endometrial renewal, inducing decidualization and the recruitment of iTregs that accompany the implantation period. In the present study we used an *in vitro* model of decidualization with a human endometrial stromal cell line (HESC) stimulated with progesterone and LPS simulating the inflammatory response during implantation.

Materials and Methods

Human Endometrial Stromal Cells (HESC)

Immortalized Human Endometrial Stromal cell line HESC described by Krikun et al. were maintained in DMEM-F12 supplemented with 10% FCS and 2mM glutamine (Krikun *et al.* 2004). For the different assays, HESC cells were cultured in 24-well plates until they reached 70% confluence. Different combinations of VIP (10^{-7}M), LPS (100 ng/ml), Progesterone (P4, 10^{-6}M, the physiologic concentration reported at the feto-maternal interface (31) and VIP-antagonist (ANT, 10^{-5}M) were added for 24 h.

Conditioned media (CM): HESC cells were cultured in DMEM-F12 10% FCS and overnight supernants were collected and maintained at -20°C until use.

Decidualization: HESC cells were cultured in 24 wells-plate with DMEM-F12 10% FCS in the presence of VIP (10^{-6}M-10^{-8}M) o medroxiprogesterone MPA (10^{-8}M)- dibutyryl cAMP (2,5 10^{-3}M) for 8 days, changing half of the culture media every 48 hours and then used in the assays described below.

Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from fertile, non-pregnant women who had two or more previous normal pregnancies without any miscarriage. The "Investigation and Ethics Committee" from the Argentinean Society of Gynecological and Reproductive Endocrinology (SAEGRE) has approved this study and all the patients provided their written consent to participate in it.

PBMCs were isolated from heparinized peripheral blood by a density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were extensively washed and resuspended in RPMI 1640 (Life Technologies Grand Island, NY) supplemented with 10% human AB serum, 2mM glutamine and 1% penicillin-streptomycin.

***In vitro* differentiation of iTregs**

In vitro differentiation was performed as previously described (Ramhorst *et al.* 2012). Briefly, naïve CD4 T cells were isolated from fertile women-PBMCs by negative depletion using the Easy Sep Kit® and following manufacturer recommendations. The recovered naïve CD4 T cells were cultured in precoated plates with anti-CD3 (10 µg/ml, BD-Pharmigen, Franklin Lakes, NJ, USA) + anti-CD28 (1 µg/ml, BD-Pharmigen, Franklin Lakes, NJ, USA) and maintained with media supplemented with IL-2 (2 ng/ml, Peprotech, USA) and recombinant TGFβ (10 ng/ml, R&D System, MN, USA). Media was changed every 48 hours and after 5 days of culture we obtained 26±4% of CD4+FOXP3+ cells.

Real Time PCR

We evaluated the chemokines IL-8, MCP-1 and RANTES as well as VIP and its receptors VPAC1 and VPAC2, and decidualization markers KLF13 and IGFBP1 expression in HESC cells under different stimuli combinations. After 24 hours of stimulation, total RNA was isolated following manufacturer recommendations with Trizol reagent (Life Technologies, Grand Island, NY, USA), cDNAs were generated from 1µg or RNA using a MMLV reverse transcriptase, RNAsin RNAse inhibitor and oligo (dT) kit (Clontech; Palo Alto, CA, USA) and stored at -20°C for batch analysis. Sample volume was

increased to 25 µl with the solution containing 50 mM KCl; 10mM Tris (pH 8.3); 1.5 mM $MgCl_2$; 0.1 µM forward and reverse primers (described in Table I), 1 U Taq polymerase in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA) and 1:30000 dilution of SybrGreen. The PCR programs used were an initial denaturalization at 95˚C for 5min, followed by 35 cycles of 95˚C for 20sec, 20sec at melting temperature specified in Table I and 20sec at 72˚C. SybrGreen fluorescence was measure at the end of each cycle. A final elongation at 72˚C for 10 min was also performed. Realtime PCR was performed on a Bio-Rad iQ5 Realtime PCR system. Results were expressed as arbitrary units normalized to GAPDH expression.

Flow-cytometry analysis

Intracellular staining for FOXP3 detection

The flow cytometric analysis was performed according to the manufacturer's instructions (Human Regulatory T cell staining kit, eBioscience, San Diego, CA). After migration, cells were recovered from the lower compartment, washed and then incubated with the fixation/permeabilization buffer for 1 hour. After washing, unspecific sites were blocked by adding 2 µl (2% final) normal rat serum, in approximately 100 µl for 15 min. Then cells were incubated with the anti-human FOXP3 (PCH101) antibody or rat IgG2a isotype control for at least 30 min at 4ºC. Finally, cells were washed with permeabilization buffer and analyzed.

Intracellular staining for VIP detection

HESC cells were stimulated with different concentrations of P4 (10^{-5}M, 10^{-6}M and 10^{-7}M) over 24 hours and incubated with Stop Golgi in the last 4 hours of culture following manufacturer's instructions (Becton Dickinson, San José, CA), to promote intracellular accumulation. To assess VIP production, HESC cells recovered after TrypLE (Invitrogen) treatment were washed by PBS, fixed and permeabilized with the citofix/Perm kit (at manufacturer´s recommended concentrations, Becton Dickinson, San José, CA). After washing, permeabilized cells were incubated for 30 min with rabbit anti-VIP Ab (Peninsula-Bachem Inc, San Carlos, CA, USA) then washed and incubated with FITC-conjugated anti-rabbit Ab (Santa Cruz, Palo Alto, CA). Cells were then washed with PBS-2% FCS to

allow membrane closure. Ten thousand events were acquired in a FACSAria II cytometer® and results were analyzed using the WinMDI software®. Negative control samples were incubated in parallel with an irrelevant, isotype-matched Ab. Results for positive cells are expressed as the mean intensity fluorescence (MIF) compared with the same cells cultured in complete media.

Migration assays

We evaluated the migration of the FOXP3+ cells using the different CM as stimuli obtained from HESC cells cultured in the presence P4, LPS, VIP or VIP-antagonist. An anti-RANTES neutralizing antibody (1 µg/ml, R&D System, MN, USA) was added during the assay to evaluate the chemokine role. After differentiation the naïve T cells were seeded in 8µm-inserts (4 x 10^4 cells/insert) (BD Falcon cell culture inserts), which then were set in a 24-well plate containing the CM from HESC cells cultured under different conditions. After 24 hours, the cells were recovered from the lower compartment and the frequency of FOXP3+ cells were quantified by FACS analysis. As a positive control, we used 20% human serum. The results are expressed as the folds of increase with respect to the positive control.

Statistical analysis

The significance of the results was analyzed by the Student´s t-test and ANOVA with Bonferroni post test for parametric anlysis of HESC cell line-samples. Mann-Whitney U-test was used for the analysis of non-parametric samples from maternal PBMCs. We used the GraphPad Prism5 software (GraphPad, San Diego, CA) and a value of *$p<0.05$ was considered significant.

RESULTS

Endometrial stromal cells express VIP/VPAC system and progesterone modulates its expression

First, we evaluated the expression of VIP and its receptors VPAC1 and VPAC2 in human endometrial stromal cells (HESC cell line). As depicted in Figure 1A, VIP and VPAC1 are constitutively expressed in stroma cells. Since progesterone (P4) has modulatory effects on endometrial cell differentiation and function at early pregnancy, we evaluated whether it affected VIP/VPAC system expression in HESC. For that purpose HESC cells were cultured at 70% of confluence in the absence or presence of P4 (10^{-6}M) and we observed that P4 significantly increased

VIP expression in HESC cells while VPAC1 was not modulated as determined by RTqPCR (Figure 1A). This result was confirmed by performing a progesterone concentration-response curve and determining median intensity fluorescence by flow cytometry, and we observed that P4 significanlty increased VIP intracellular production with a peak at 10^{-6}M Figure 1B. The mean intensity fluorescence of VIP in HESC treated with different P4 concentrations is also shown as a representative histogram. VPAC2 expression was not detected in HESC cells under these conditions.

VIP induces chemokine expression

Our next objective was to determine the effect of VIP on the expression of chemokines involved in leukocyte recruitment toward endometrial stromal cells. In addition, we evaluated the effect of LPS as a proinflammatory stimulus. Hence, the expression of the chemokines RANTES (CCL5) involved in T cell recruitment, IL-8 (CXCL8) involved in neutrophils recruitment, and MCP-1 (CCL2), one of the main chemokines involved in monocyte/macrophage recruitment were evaluated by RTqPCR in HESC cells stimulated or not with VIP (10^{-7}M) and LPS (100 ng/ml). As shown in figures 2A, B and C, LPS increased RANTES, IL-8 and MCP-1 expression. VIP by itself did not have a significant effect on cytokine production by HESCs; however, the combination of LPS and VIP further enhanced LPS-induced RANTES expression.

Progesterone induces RANTES expression through a VIP pathway

Since RANTES expression was further increased in the presence of VIP and LPS, and P4 induced endogenous VIP production, we investigated whether RANTES expression was modulated by endogenous VIP on HESC cells. Therefore, HESC cells were cultured in the absence or presence of P4, LPS (100 ng/ml) and VIP-antagonist to evaluate the relevance of the endogenous VIP. We could observe that VIP-induced RANTES expression in the presence of LPS was prevented by VIP antagonist (Figure 3A). Progesterone also induced RANTES expression and this was far more pronounced in the presence of LPS. VIP antagonist prevented the increase of RANTES expression

mediated by P4 and LPS, suggesting that RANTES induction involved a VIP-mediated pathway (Figure 3A).

We then investigated the modulation of one transcription factor involved in RANTES expression as the Kruppel-Like Factor 13 (KLF13) (Song *et al.* 2002, Pabona *et al.* 2010). HESC cells were cultured in the absence or presence of P4, VIP, LPS and VIP antagonist and then KLF13 expression was evaluated by RTqPCR. As shown in Figure 3B, P4 and VIP in the presence of LPS significantly increased KLF13 expression. VIP antagonist prevented the increase of KLF13 expression induced only by VIP and LPS.

Endometrial stromal cells specifically recruit iTregs through RANTES production

Our next step was to determine if HESC cells have the ability to attract iTregs. Human Treg cells were differentiated from naïve CD45RA+CCR7+ cells obtained from fertile women PBMCs cultured with IL-2 and TGF-β over 5 days, as described in the Materials and Methods section. We then performed migration assays using a multi-chamber system. *In vitro* differentiated iTregs were seeded onto 8µm pore-inserts, allowing cell migration towards the CM used as a chemotactic stimulus in the lower compartment. After 24h cells were recovered from the lower compartment and FOXP3 expression was quantified by FACS analysis. As depicted in figure 4A, the CM from HESC cells increased the frequency of FOXP3+ cells to levels similar to the migration observed in the presence of human serum (positive control). However, when the migration assay was performed in the presence of CM from HESC cells cultured in the presence of VIP antagonist, the recruitment of iTregs to the lower compartment was prevented (see Figure 4A). Moreover, addition of anti-RANTES neutralizing Ab to the CM from HESC treated with P4 and LPS also was able to prevent iTregs migration (see Figure 4A). Figure 4B shows representative dotplots with the percentage of FOXP3+ cells. We did not observe changes in the migration rate of the FOXP3 negative population under the same conditions mentioned above suggesting that RANTES participates in the specific recruitment of iTregs toward HESC cells (Figure 4C).

VIP induces decidualization of endometrial stromal cells

On the hypothesis of a potential contribution of VIP to the decidualization program, we investigated VIP direct effects on endometrial stromal cells. Therefore, HESC cells were cultured in the absence or presence of VIP (10^{-8}M to 10^{-6}M) or in the presence of medroxyprogesterone-dibutyryl cAMP, as a positive control of decidualization, and after 8 days of culture we evaluated the expression of IGFBP-1 (Insulin-like growth factor-binding protein 1, also known as Placental Protein 12), a decidualization marker. We observed that HESC cells cultured with 10^{-7}M and 10^{-6} M VIP significantly increased IGFBP-1 expression (Figure 5A). The modulation in IGFBP-1 was accompained by morphological changes that characterize the decidualization process as depicted in Figure 5B.

Since endometrial stromal cells after decidualization increased chemokine production and VIP induced the marker of decidualization IGFBP-1 on HESC cells, we wondered if VIP was also able to increase RANTES expression after cell differentiation. HESC cells were decidualized in the presence of VIP (10^{-6}M to 10^{-8}M) and after 8 days we observed a significant increase in RANTES expression (see Figure 5C). This increase was also accompanied by a significant increase in the expression of KLF13 in HESC cells, which is a RANTES transcription factor besides a decidualization marker (see Figure 5D).

Taken together, these results suggest that VIP might participate in the decidualization process not only by the induction of decidualization markers, but also by increasing RANTES production, which mediates the recruitment of iTregs.

DISCUSSION

In humans, the decidualization process involves the transformation of stromal fibroblasts into epithelioid decidual cells and the recruitment of immune cells critical for decidual development in an early inflammatory microenvironment, thus, multiple regulatory mechanisms are required to maintain

the local immune homeostasis (Wilcox *et al.* 1999, Cahouat *et al.* 2010, Terness *et al.* 2007, Yoshinaga *et al.* 2010).

In line with the strict regulation that Treg cells have in the control of the effector immune responses throughout pregnancy (Guerin *et al.* 2010, Robertson *et al.* 2009, Aluvihare *et al.* 2004, Ramhorst *et al.* 2012), we analyzed the contribution of the neuropeptide VIP to the decidualization program reflected by the increase of decidualization markers and by the recruitment of iTregs toward endometrial stromal cells as a local regulator of the implantatory inflammatory response. For that purpose, we used the HESC cell line cultured under different stimuli and iTregs differentiated from naïve CD45RA+CCR7+ obtained from fertile women's PBMCs as an *in vitro* model of interaction. HESC cells were cultured in the presence of an effective concentration of P4 (10^{-6}M), and LPS (100 ng/ml) as an inflammatory stimulus that modulated chemokine production but did not affect cell viability (Abraham *et al.* 2004b).

The results presented in this study suggest that the neuropeptide VIP, with potent anti-inflammatory and immunomodulatory effects, could contribute to the decidualization process inducing endometrial stromal cell expression of decidualization markers and the selective recruitment of iTregs toward HESC cells by increasing RANTES production under the effect of progesterone in the presence of a inflammatory microenvironment.

Our conclusions are based on several observations: First, HESC cells express VIP and its constitutive receptor VPAC1, and P4 has the ability to increase VIP production. Second, RANTES expression, one of the main chemokines involved in T cell recruitment was induced by VIP in the presence of LPS and its induction was mediated by P4. Finally, the migration assay of iTregs toward conditioned media from HESC cells revealed that the endogenous VIP production induced by P4 and LPS stimulation could selectively attract them through RANTES production, since the anti-RANTES neutralizing Ab or VIP antagonist prevented the iTregs migration.

VIP might be one of the first mediators that induces decidualization through its interaction with the VPAC1 receptor and triggering cAMP signaling in HESC cells to increase the expression of IGFBP-1 and KLF-13, both markers of decidualization accompained by morphological changes

characteristic of decidualized cells. In fact, KLF13 is not only a decidualization marker, also is a transcription factor that binds to RANTES-promoter, necessary to mediate RANTES transcription (Song *et al.* 2002). This mechanism could explain how endogenous VIP regulates RANTES expression on HESC cells thus contributing to the selective recruitment of iTregs that might allow the control of tissue damage during embryo implantation. In this sense, Nancy et al. (Nancy *et al.* 2012) recently reported that genes encoding chemokines are subject to epigenetic silencing in decidual stromal cells to restrain the attraction of Th1 and T cytotoxic profiles as a strategy to prevent potential tissue damage. In brief, the decidualization program involves many regulatory molecules that play functional roles, such as insulin-like growth factors, interleukin-1, 6, 10 and TGF-β families, the neuropeptide VIP, chemokines as RANTES with their receptors, and adhesion molecules that generate a network to control implantation processes such as trophoblast adhesion, invasion and the selective recruitment of maternal leukocyte subpopulations (Dimitriadis *et al.* 2000, Salomensen *et al.* 1999, Terness *et al.* 2007, Yoshinaga *et al.* 2010, Fraccaroli *et al.* 2011).

Interestingly, spontaneous decidualization of stromal cells occurs in the absence of pregnancy. It was proposed that cyclic endometrial decidualization followed by menstruation “preconditions“ uterine tissues for a hyperinflammatory response and oxidative stress that is in turn accompanied by deep trophoblast invasion during early pregnancy (Teklenburg *et al.* 2010a, Teklenburg *et al.* 2010b, Bronsens *et al.* 2009). Therefore, the ability of the human endometrium to generate an adequate decidual response based on successive inflammatory events might contribute to a sensitization of the uterine tissues. Under this hypothesis of repeated inflammatory events it is conceivable that a tight immune homeostatic control prior to implantation is required (Kim *et al.* 2009, Weiss *et al.* 2009). In this context the ability of HESC cells to selectively recruit iTregs might contribute to maintain immune homeostasis at early stages of implantation.

Finally, even though the research in the past few years provided a better understanding of trophoblast-endometrial interactions during the initial stage of implantation by means of various human cell experimental approaches, the identification of biomarkers with clinical utility for patients with implantation failures is still open.

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344 **Authors' contributions:** CPL and RR designed the study and wrote the manuscript. EG carried out

345 all the experiments with HESC cells and decidualization, the differentiation of iTregs and the co

346 cultures, the migration assays. DP and MA helped with RT-PCRs data analyses and interpretation.

347 GM supervised the study and discuss the results . All authors read and approved the final manuscript.

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References

Abrahams VM, Kim YM, Straszewski SL, Romero R & Mor G 2004a Macrophages and apoptotic cell clearance during pregnancy. *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **51** 275–282. (doi:10.1111/j.1600-0897.2004.00156.x)

Abrahams VM, Bole-Aldo P, Kim YM, Straszewski-Chavez SL, Chaiworapongsa T, Romero R & Mor G 2004b Divergent trophoblast responses to bacterial products mediated by TLRs. *Journal of Immunology (Baltimore, Md.□: 1950)* **173** 4286–4296.

Aluvihare VR, Kallikourdis M & Betz AG 2004 Regulatory T cells mediate maternal tolerance to the fetus. *Nature Immunology* **5** 266–271. (doi:10.1038/ni1037)

Bromley SK, Mempel TR & Luster AD 2008 Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nature Immunology* **9** 970–980. (doi:10.1038/ni.f.213)

Brosens JJ, Parker MG, McIndoe A, Pijnenborg R & Brosens IA 2009 A role for menstruation in preconditioning the uterus for successful pregnancy. *American Journal of Obstetrics and Gynecology* **200** 615.e1–6. (doi:10.1016/j.ajog.2008.11.037)

Catalano RD, Critchley HO, Heikinheimo O, Baird DT, Hapangama D, Sherwin JRA, Charnock-Jones DS, Smith SK & Sharkey AM 2007 Mifepristone induced progesterone withdrawal reveals novel regulatory pathways in human endometrium. *Molecular Human Reproduction* **13** 641–654. (doi:10.1093/molehr/gam021)

Chaouat G, Petitbarat M, Dubanchet S, Rahmati M & Ledée N 2010 Tolerance to the foetal allograft? *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **63** 624–636. (doi:10.1111/j.1600-0897.2010.00832.x)

Couvineau A & Laburthe M 2012 VPAC receptors: structure, molecular pharmacology and interaction with accessory proteins. *British Journal of Pharmacology* **166** 42–50. (doi:10.1111/j.1476-5381.2011.01676.x)

Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T & Wang H 2004 Molecular cues to implantation. *Endocrine Reviews* **25** 341–373. (doi:10.1210/er.2003-0020)

Dimitriadis E, Nie G, Hannan NJ, Paiva P & Salamonsen LA 2010 Local regulation of implantation at the human fetal-maternal interface. *The International Journal of Developmental Biology* **54** 313–322. (doi:10.1387/ijdb.082772ed)

Ekström J, Månsson B & Tobin G 1983 Vasoactive intestinal peptide evoked secretion of fluid and protein from rat salivary glands and the development of supersensitivity. *Acta Physiologica Scandinavica* **119** 169–175. (doi:10.1111/j.1748-1716.1983.tb07322.x)

Fraccaroli L, Alfieri J, Leiros CP & Ramhorst R 2009a Immunomodulatory effects of chemokines during the early implantation window. *Frontiers in Bioscience (Elite Edition)* **1** 288–298.

Fraccaroli L, Alfieri J, Larocca L, Calafat M, Mor G, Leirós CP & Ramhorst R 2009b A potential tolerogenic immune mechanism in a trophoblast cell line through the activation of chemokine-induced T cell death and regulatory T cell modulation. *Human Reproduction (Oxford, England)* **24** 166–175. (doi:10.1093/humrep/den344)

Fraccaroli L, Alfieri J, Larocca L, Calafat M, Roca V, Lombardi E, Ramhorst R & Leirós CP 2009c VIP modulates the pro-inflammatory maternal response, inducing tolerance to trophoblast cells. *British Journal of Pharmacology* **156** 116–126. (doi:10.1111/j.1476-5381.2008.00055.x)

Fraccaroli L, Grasso E, Zeitler E, Lombardi E, Gogorza S, Etchepareborda JJ, Nagle C, Cortelezzi M, Pérez Leirós C & Ramhorst R 2011 Modulation of maternal LIF producers T cells by trophoblast and paternal antigens. *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **65** 133–145. (doi:10.1111/j.1600-0897.2010.00890.x)

Gellersen B & Brosens J 2003 Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. *The Journal of Endocrinology* **178** 357–372.

Gellersen B, Brosens IA & Brosens JJ 2007 Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Seminars in Reproductive Medicine* **25** 445–453. (doi:10.1055/s-2007-991042)

Gomez-Lopez N, Guilbert LJ & Olson DM 2010 Invasion of the leukocytes into the fetal-maternal interface during pregnancy. *Journal of Leukocyte Biology* **88** 625–633. (doi:10.1189/jlb.1209796)

Gonzalez-Rey E, Anderson P & Delgado M 2007 Emerging roles of vasoactive intestinal peptide: a new approach for autoimmune therapy. *Annals of the Rheumatic Diseases* **66** **Suppl 3** iii70–6. (doi:10.1136/ard.2007.078519)

Guerin LR, Prins JR & Robertson S a 2009 Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Human Reproduction Update* **15** 517–535. (doi:10.1093/humupd/dmp004)

Kim MJ, Romero R, Kim CJ, Tarca AL, Chhauy S, LaJeunesse C, Lee D-C, Draghici S, Gotsch F, Kusanovic JP *et al.* 2009 Villitis of unknown etiology is associated with a distinct pattern of chemokine up-regulation in the feto-maternal and placental compartments: implications for conjoint maternal allograft rejection and maternal anti-fetal graft-versus-host disease. *Journal of Immunology (Baltimore, Md.□: 1950)* **182** 3919–3927. (doi:10.4049/jimmunol.0803834)

King AE & Critchley HOD 2010 Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. *The Journal of Steroid Biochemistry and Molecular Biology* **120** 116–126. (doi:10.1016/j.jsbmb.2010.01.003)

Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, Rahman M, Caze R, Qumsiyeh M & Lockwood CJ 2004 A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology* **145** 2291–2296. (doi:10.1210/en.2003-1606)

425 Leceta J, Gomariz RP, Martinez C, Carrión M, Arranz A & Juarranz Y 2007 Vasoactive
426 intestinal peptide regulates Th17 function in autoimmune inflammation.
427 *Neuroimmunomodulation* **14** 134–138. (doi:10.1159/000110636)

428 Mesiano S, Wang Y & Norwitz ER 2011 Progesterone receptors in the human pregnancy
429 uterus: do they hold the key to birth timing? *Reproductive Sciences (Thousand Oaks,*
430 *Calif.)* **18** 6–19. (doi:10.1177/1933719110382922)

431 Nancy P, Tagliani E, Tay C-S, Asp P, Levy DE & Erlebacher A 2012 Chemokine gene
432 silencing in decidual stromal cells limits T cell access to the maternal-fetal interface.
433 *Science (New York, N.Y.)* **336** 1317–1321. (doi:10.1126/science.1220030)

434 Pabona JMP, Zeng Z, Simmen F a & Simmen RCM 2010 Functional differentiation of
435 uterine stromal cells involves cross-regulation between bone morphogenetic protein 2
436 and Kruppel-like factor (KLF) family members KLF9 and KLF13. *Endocrinology* **151**
437 3396–3406. (doi:10.1210/en.2009-1370)

438 Paria BC, Reese J, Das SK & Dey SK 2002 Deciphering the cross-talk of implantation:
439 advances and challenges. *Science (New York, N.Y.)* **296** 2185–2188.
440 (doi:10.1126/science.1071601)

441 Pérez Leirós C & Ramhorst R 2013 Tolerance induction at the early maternal-placental
442 interface through selective cell recruitment and targeting by immune polypeptides.
443 *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **69** 359–368.
444 (doi:10.1111/aji.12087)

445 Ramhorst R, Patel R, Corigliano A, Etchepareborda JJ, Fainboim L & Schust D 2006
446 Induction of maternal tolerance to fetal alloantigens by RANTES production. *American*
447 *Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **56** 302–311.
448 (doi:10.1111/j.1600-0897.2006.00430.x)

449 Ramhorst R, Gutiérrez G, Corigliano A, Junovich G & Fainboim L 2007 Implication of
450 RANTES in the modulation of alloimmune response by progesterone during
451 pregnancy. *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)*
452 **57** 147–152. (doi:10.1111/j.1600-0897.2006.00458.x)

453 Ramhorst R, Fraccaroli L, Aldo P, Alvero AB, Cardenas I, Leirós CP & Mor G 2012
454 Modulation and recruitment of inducible regulatory T cells by first trimester
455 trophoblast cells. *American Journal of Reproductive Immunology (New York, N.Y.□:*
456 *1989)* **67** 17–27. (doi:10.1111/j.1600-0897.2011.01056.x)

457 Robertson S a, Prins JR, Sharkey DJ & Moldenhauer LM 2013 Seminal fluid and the
458 generation of regulatory T cells for embryo implantation. *American Journal of*
459 *Reproductive Immunology (New York, N.Y.□: 1989)* **69** 315–330.
460 (doi:10.1111/aji.12107)

461 Salamonsen LA & Woolley DE 1999 Menstruation: induction by matrix metalloproteinases
462 and inflammatory cells. *Journal of Reproductive Immunology* **44** 1–27.

Song A, Patel A, Thamatrakoln K, Liu C, Feng D, Clayberger C & Krensky AM 2002 Functional domains and DNA-binding sequences of RFLAT-1/KLF13, a Krüppel-like transcription factor of activated T lymphocytes. *The Journal of Biological Chemistry* **277** 30055–30065. (doi:10.1074/jbc.M204278200)

Spong CY, Lee SJ, McCune SK, Gibney G, Abebe DT, Alvero R, Brenneman DE & Hill JM 1999 Maternal regulation of embryonic growth: the role of vasoactive intestinal peptide. *Endocrinology* **140** 917–924. (doi:10.1210/endo.140.2.6481)

Stoikos CJ, Harrison CA, Salamonsen LA & Dimitriadis E 2008 A distinct cohort of the TGFbeta superfamily members expressed in human endometrium regulate decidualization. *Human Reproduction (Oxford, England)* **23** 1447–1456. (doi:10.1093/humrep/den110)

Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landles C *et al.* 2010a Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PloS One* **5** e10258. (doi:10.1371/journal.pone.0010258)

Teklenburg G, Salker M, Heijnen C, Macklon NS & Brosens JJ 2010b The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. *Molecular Human Reproduction* **16** 886–895. (doi:10.1093/molehr/gaq079)

Terness P, Kallikourdis M, Betz AG, Rabinovich GA, Saito S & Clark DA 2007 Tolerance signaling molecules and pregnancy: IDO, galectins, and the renaissance of regulatory T cells. *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **58** 238–254. (doi:10.1111/j.1600-0897.2007.00510.x)

Van Voorhis BJ, Anderson DJ & Hill JA 1989 The effects of RU 486 on immune function and steroid-induced immunosuppression in vitro. *The Journal of Clinical Endocrinology and Metabolism* **69** 1195–1199. (doi:10.1210/jcem-69-6-1195)

Weiss G, Goldsmith LT, Taylor RN, Bellet D & Taylor HS 2009 Inflammation in reproductive disorders. *Reproductive Sciences (Thousand Oaks, Calif.)* **16** 216–229. (doi:10.1177/1933719108330087)

Wilcox AJ, Baird DD & Weinberg CR 1999 Time of implantation of the conceptus and loss of pregnancy. *The New England Journal of Medicine* **340** 1796–1799. (doi:10.1056/NEJM199906103402304)

Yoshinaga K 2010 Research on Blastocyst Implantation Essential Factors (BIEFs). *American Journal of Reproductive Immunology (New York, N.Y.□: 1989)* **63** 413–424. (doi:10.1111/j.1600-0897.2010.00853.x)

Legends to Figures

Figure 1: VIP/VPAC system on HESC cells. (A) HESC cells at 70% of confluence in a 24 well flat-bottom plate were cultured in the absence or presence of P4 (10^{-6}M). After 24 hours, HESC cells were

recovered and the expression of VIP and its receptors VPAC1 was evaluated by RTqPCR. Result shown is representative of five others run similarly and bars at the right side represent VIP and VPAC1 mean expression relative to GAPDH ± S.E.M from 5 independent experiments (*p<0.05, Student t-test). **(B):** HESC cells also were cultured in the presence of P4 at different concentrations during 24 hours. Recovered cells were permeabilized and intracellularly stained with anti-VIP mAb. Results are expressed as the Mean Intensity Flourescence (MIF) and represent mean ± SEM of 3 independent experiments (*p<0.05, Student t-test). The right panel shows a representative histogram profile of one of 3 similar experiments.

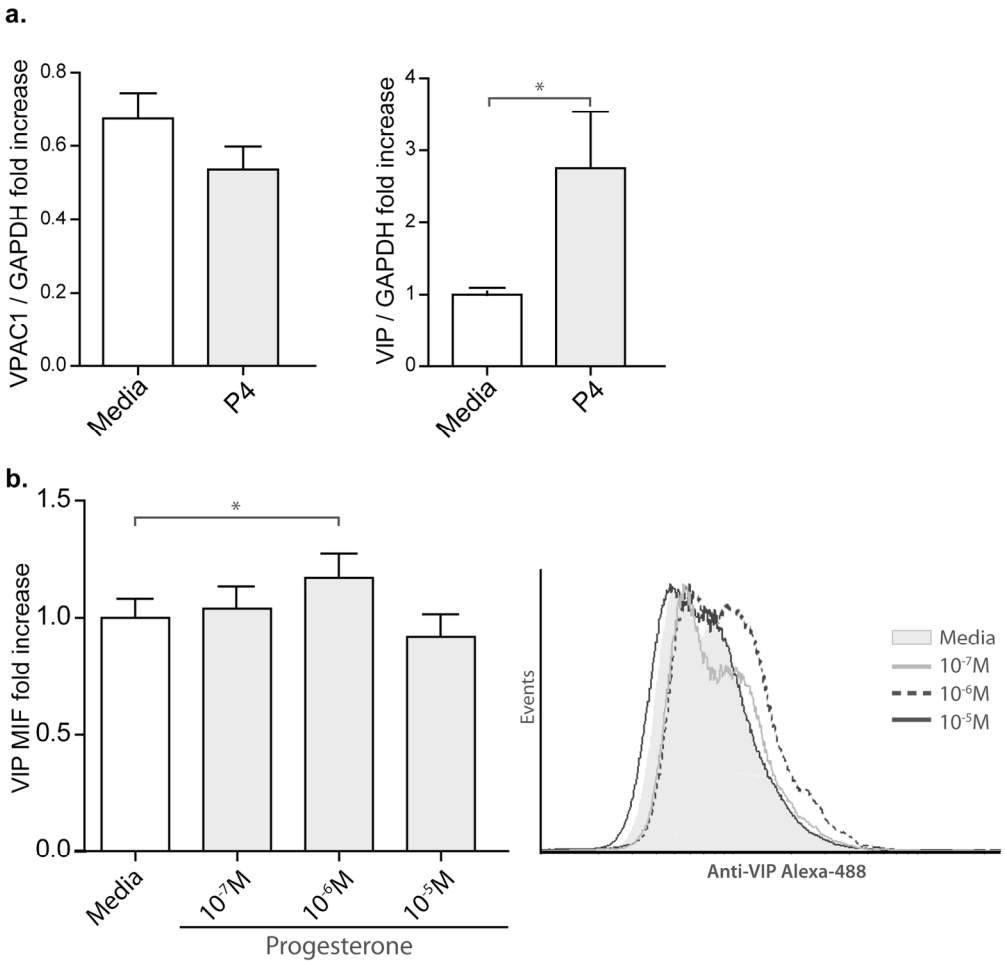
Figure 2: Induction of chemokines by LPS and VIP. HESC cells at 70% of confluence in a 24 well flat-bottom plate, were cultured in the absence or presence of VIP (10^{-7}M) and LPS (100 ng/ml). After 24 hours, HESC cells were recovered and the expression of **(A):** RANTES (CCL5), **(B):** IL-8 (CXCL8) and **(C):** MCP-1 (CCL2) was evaluated byRTqPCR. Bars represent chemokine mean expression relative to GAPDH ± S.E.M from 5 independent experiments (*p<0.05, Student t-test).

Figure 3: Progesterone induced RANTES expression through a VIP pathway. HESC cells at 70% of confluence in a 24 well flat-bottom plate, were stimulated with different combinations of VIP (10^{-7}M), P4 (10^{-6}M), VIP-antagonist (ANT: 10^{-5}M) and LPS (100 ng/ml). After 24 hours, HESC cells were recovered and the expression of RANTES **(A)** and KLF13 **(B)** were evaluated by RTqPCR. Bars represent RANTES or KLF13 mean expression relative to GAPDH ± S.E.M relativized to LPS stimuli from 6 independent experiments (*p<0.05, ANOVA, post test Bonferroni).

Figure 4. Modulation of iTregs migration. The iTregs were differentiated *in vitro* obtained from CD4+ naïve T cells as described in Material and Methods. An 8µm transwell system was used for migration assay. The iTregs were seeded in the upper compartment and CM obtained from HESC cells under different stimuli (cultured in presence of VIP (10^{-7}M), P4 (10^{-6}M), VIP-antagonist (ANT: 10^{-5}M) and LPS (100 ng/ml)) in the lower compartment in the presence or not of anti-RANTES (αRANTES) neutralizing antibody. The migration was evaluated by flow cytometry as total number of FOXP3+

cells. **(A)** The results are expressed as the folds of increase with respect to the positive control (AB human-serum) from 3 independent experiments using different maternal PBMCs (Mann Whitney test *p<0.05) and **(B)** shows representatives dot plots profile with the percentage of FOXP3+ cells.

Figure 5: VIP induced decidualization of endometrial stromal cells. HESC cells were cultured in the absence or presence of VIP (10^{-6}M to 10^{-8}M) or in the presence of MPA-dibutyryl cAMP as positive control, and after 8 days of culture we evlauted the expression of IGFBP-1 **(A)**, the morphologic changes after 24 days of cultured **(B),** the expression of RANTES **(C)** and KLF13 **(D**) by RTqPCR**.** Bars represent mean expression relative to GAPDH ± S.E.M from 3 independent experiments (*p<0.05, ANOVA, post test Bonferroni).



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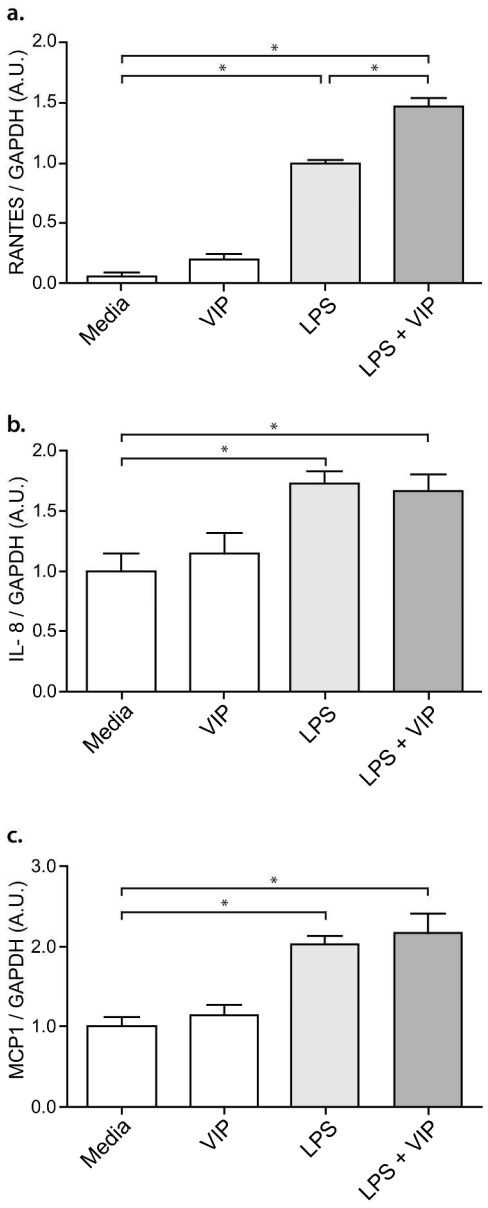

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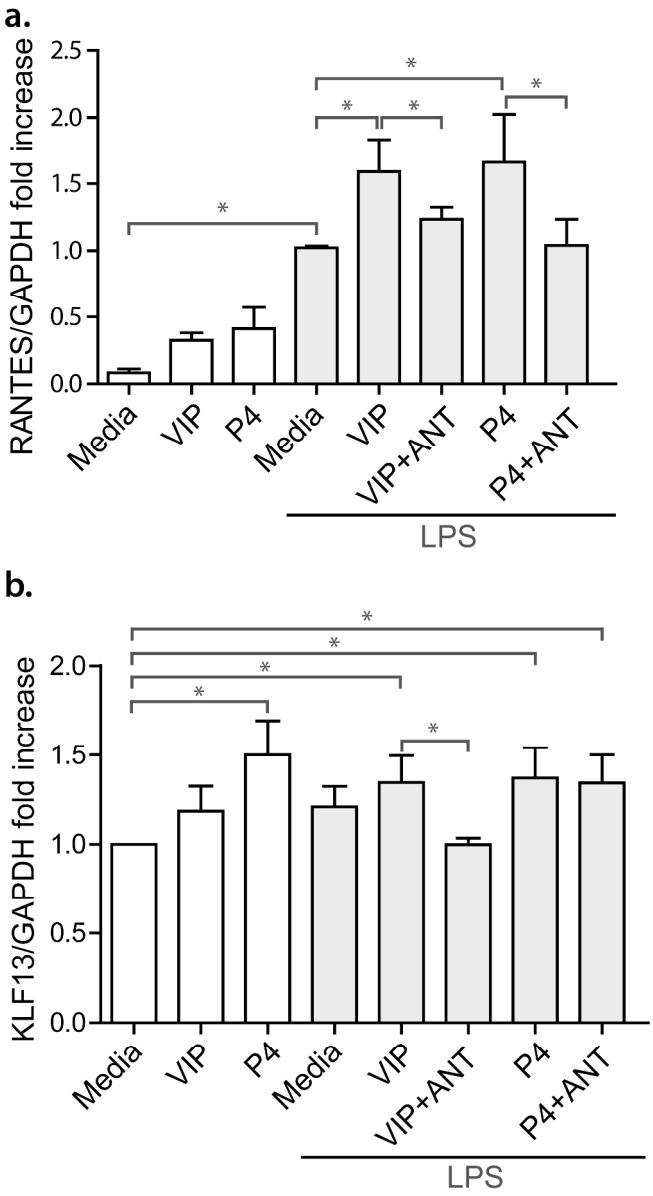

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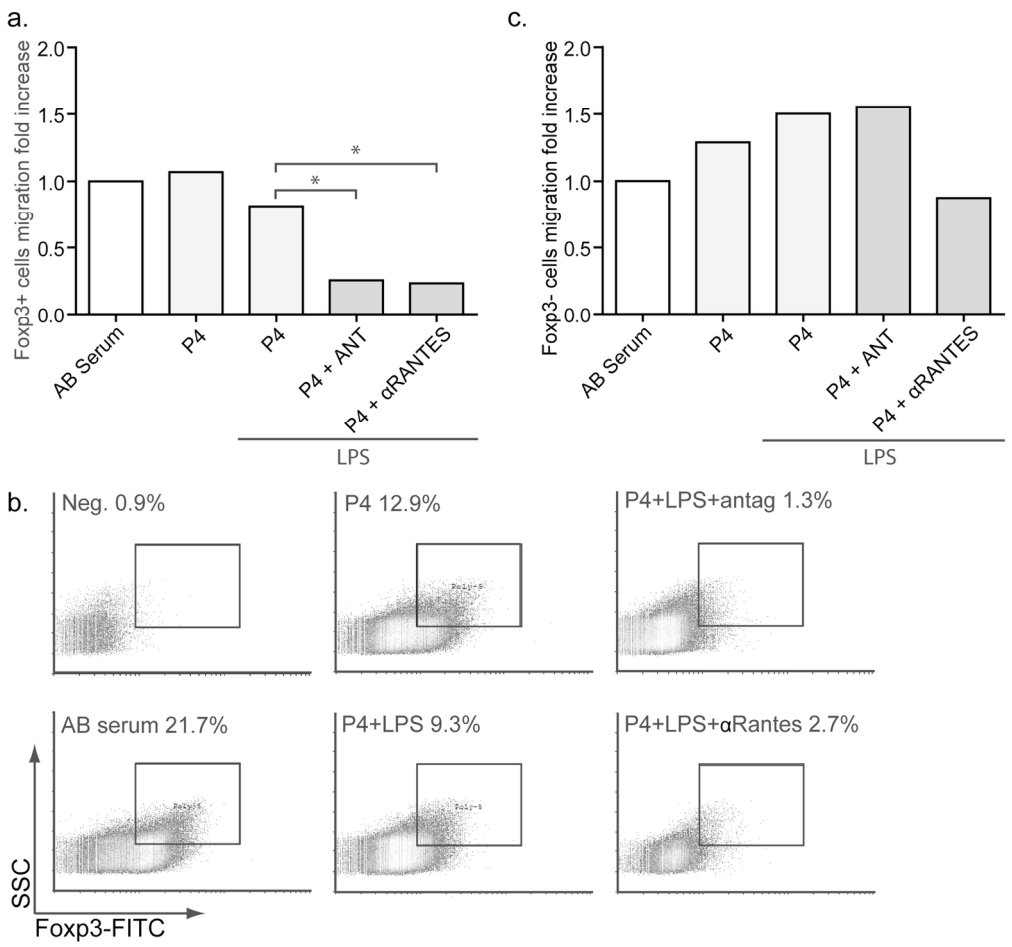

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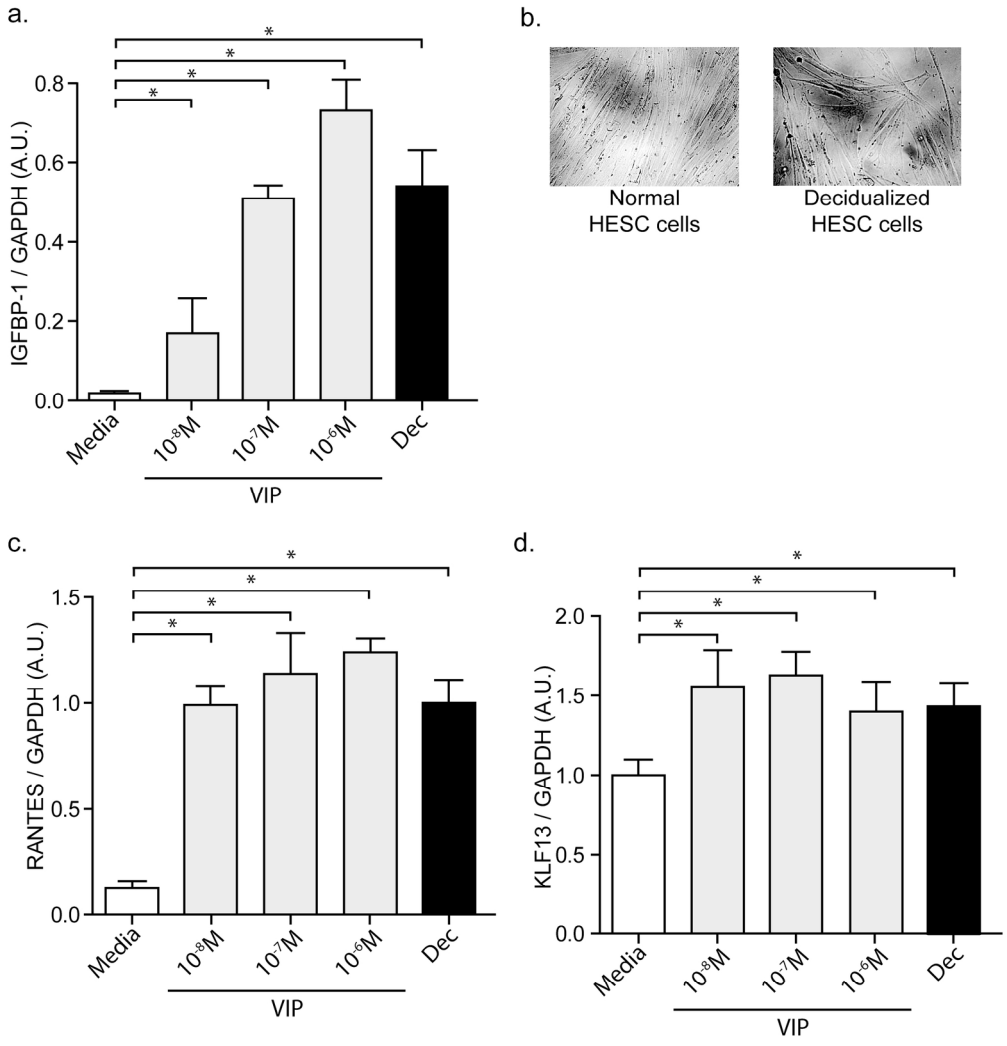

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Table I: PCR primers

Gene	Primer (5'-3')	Product length	Tm (˚C)
VIP	Fw TACAGGGCACCTTCTGCTCT	198 bp	57
	Rv CAAGAGTTTACTGAAGTCACT		
VPAC1	Fw CCCCTGGGTCAGTCTGGTG	100 bp	58
	Rv GAGACCTAGCATTCGCTGGTG		
VPAC2	Fw CCAGATGTCGGCGGCAACG	114 bp	56
	Rv GCTGATGGGAAACACGGCAAAC		
IL-8	Fw CCAACACAGAAATTATTGTAAAGC	163 bp	62
	Rv CACTGGCATCTTCACTGATTC		
MCP1	Fw CAGCAGCAAGTGTCCCAAAG	146 bp	64
	Rv GAGTGAGTGTTCAAGTCTTCGG		
RANTES	Fw TGCTGCTTTGCCTACATTGC	95 bp	64
	Rv AAGACGACTGCTGGGTTGG		
KLF13	Fw TTCGGTGGTTCCTTGGTGACTGG	169 bp	61
	Rv TGGACCCTTGGATTCTGCCTTGG		